Amendments to the Specification:

Please amend paragraph 2 on page 2 as follows:

(60/185,987) Chloroplasts are prokaryotic compartments inside eukaryotic cells. Since the transcriptional and translational machinery of the chloroplast is similar to *E. coli* (Brixey et al., 1997), it is possible to express prokaryotic genes at very high levels in plant chloroplasts than in the nucleus. In addition, plant cells contain up to 50,000 copies of the circular plastid genome (Bendich 1987) which may amplify the foreign gene like a "plasmid in the plant cell," thereby enabling higher levels of expression[[ ]]. Therefore, chloroplasts are an ideal choice for expression of recombinant proteins that are currently expressed in *E. coli* (such as insulin, human serum albumin, vaccines, antibodies, etc.). We exploited the chloroplast transformation approach to express a pharmacological protein that is of no value to the plant to demonstrate this concept[[,]]. GVGVP (SEQ ID NO:20) gene has been synthesized with a codon preferred for prokaryotic (EG121) or eukaryotic (TG131) expression. Based on transcript levels, chloroplast expression of this polymer was a hundred-fold higher than nuclear expression in transgenic plants (Guda et al., 1999). Recently, we observed 16.966-fold more tps 1 transcripts in chloroplast transformants than the highly expressing nuclear transgenic plants (Lee et al. 2000, in review).

Please amend the first full paragraph on page 5 as follows:

(60/263,668) Another major cost of insulin production is purification. Chromatography accounts for 30% of operating expenses and 70% of equipment in production of insulin (Petridis et al. 1995). Therefore, new approaches are needed to minimize or eliminate chroma[[-]]tography in insulin production. One such approach is the use of GVGVP (SEQ ID NO:20) as a fusion protein to facilitate single step purification without the use of chromatography. GVGVP (SEQ ID NO:20) is a Protein Based Polymer (PBP) made from synthetic genes. At lower temperatures this polymer exists as more extended molecules. Upon raising the temperature above the transition

range, polymer hydrophobically folds into dynamic structures called β-spirals that further aggregate by hydrophobic association to form twisted filaments (Urry, 1991; Urry et al., 1994). Inverse temperature transition offers several advantages. It facilitates scale up of purification from grams to kilograms. Milder purification condition requires only a modest change in temperature and ionic strength. This should also facilitate higher recovery, faster purification and high volume processing. Protein purification is generally the slow step (bottleneck) in pharmaceutical product development. Through exploitation of this reversible inverse temperature transition property, simple and inexpensive extraction and purification may be performed. The temperature at which the aggregation takes place can be manipulated by engineering biopolymers containing varying numbers of repeats and changing salt concentration in solution (McPherson et al., 1996). Chloroplast mediated expression of insulin-polymer fusion protein should eliminate the need for the expensive fermentation process as well as reagents needed for recombinant protein purification and downstream processing.

Please amend the first full paragraph on page 6 as follows:

(60/263,668) Taken together, low levels of expression of human proteins in nuclear transgenic plants, and difficulty in folding, assembly/processing of human proteins in *E._coli* should make chloroplasts an alternate compartment for expression of these proteins. Production of human proteins in transgenic chloroplasts should also dramatically lower the production cost. Large-scale production of insulin in tobacco in conjunction with an oral delivery system can be a powerful approach to provide treatment to diabetes patients at an affordable cost and provide tobacco farmers alternate uses for this hazardous crop. Therefore, it is first advantageous to use poly(GVGVP) (SEQ ID NO:1) as a fusion protein to enable hyper-expression of insulin and accomplish rapid one step purification of the fusion peptide utilizing the inverse temperature transition properties of this polymer. It is further advantageous to develop insulin-CTB fusion protein for oral delivery in nicotine free edible tobacco (LAMD 605).

Please amend paragraph 2 on page 6 as follows:

This invention synthesizes high value pharmaceutical proteins in transgenic plants by chloroplast expression for pharmaceutical protein production. Chloroplasts are suitable for this purpose because of their ability to process eukaryotic proteins, including folding and formation of disulfide bridges, thereby eliminating the need for expensive post-purification processing. Tobacco is an ideal choice for this purpose because of its large biomass, ease of scale-up (million seeds per plant) and genetic manipulation. We use poly(GVGVP) (SEQ ID NO:1) as a fusion protein to enable hyper-expression of insulin and accomplish rapid one step purification of fusion peptides utilizing the inverse temperature transition properties of this polymer. We also use insulin-CTB fusion protein in chloroplasts of nicotine free edible tobacco (LAMD 605) for oral delivery to NOD mice.

Please amend paragraph 6 on page 9 as follows:

Figure 34 shows a comparison of the DNA sequences of native human proinsulin (SEQ ID NO:16) and plastid modified proinsulin (SEQ ID NO:17).

Please amend paragraph 3 on page 10 as follows:

Figure 42A shows IGF-I native sequence coding for the mature protein (SEQ ID NO:18).

Please amend paragraph 4 on page 10 as follows:

Figure 42B shows IGF-I optimized sequence according to chloroplast preferred codon usage (SEQ ID NO:19).

Please insert the following paragraphs after paragraph 5 on page 10:

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is an amino acid encompassing 1 to 250 GVGVP repeats. GVGVP is a protein-based polymer (PBP) made from synthetic genes.

SEQ ID NO:2 is an illustrative endoplasmic reticulum retention signal amino acid.

SEQ ID NO:3 is a flexible hinge tetrapeptide.

SEQ ID NO:4 is a polynucleotide primer used according to the subject invention.

SEQ ID NO:5 is a polynucleotide primer used according to the subject invention.

SEQ ID NO:6 is a polynucleotide primer used according to the subject invention.

SEQ ID NO:7 is a polynucleotide primer used according to the subject invention.

SEQ ID NO:8 is intentionally skipped to avoid renumbering sequences.

SEQ ID NO:9 is a synthetic peptide.

SEQ ID NO:10 is the cleavage site recognized for the TEV protease.

SEQ ID NO:11 is a cleavage site recognized by Thrombin.

SEQ ID NO:12 is a 6-His tag.

SEQ ID NO:13 is the polynucleotide PCR primer 3P used according to the subject invention.

SEQ ID NO:14 is a polynucleotide PCR primer 3M used according to the subject invention.

SEQ ID NO:15 is a protein of *E. coli.*

SEQ ID NO:16 is a polynucleotide sequence from Homo sapiens.

SEQ ID NO:17 is a chloroplast modified proinsulin sequence.

SEQ ID NO:18 is an IGF-I native polynucleotide sequence from Homo sapiens coding for the mature protein.

SEQ ID NO:19 is an IGF-I optimized polynucleotide sequence according to chloroplast preferred codon usage.

SEQ ID NO:20 is a protein-based polymer (PBP) made from synthetic genes.

SEQ ID NO:21 is an elastic PBP $(GVGVP)_{121}$.

SEQ ID NO:22 is an elastic PBP GVGVP 50-mer.

SEQ ID NO:23 is the RBS sequence GAAGGAG.

SEQ ID NO:24 is an elastic PBP $GVGVP_{40}$.

SEQ ID NO:25 is an elastic PBP $GVGVP_{20}$.

SEQ ID NO:26 is the chloroplast preferred Ribosome Binding Site (RBS) Shine-Dalgarno sequence GGAGG.

Please amend paragraph 6 on page 10 as follows:

(60/[[,]]263,668) A remarkable feature of chloroplast genetic engineering is the observation of exceptionally large accumulation of foreign proteins in transgenic plants. This can be as much as 46% of CRY protein in total soluble protein, even in bleached old leaves (DeCosa et al. 2001). Stable expression of a pharmaceutical protein in chloroplasts was first reported for GVGVP (SEQ ID NO:20), a protein based polymer with varied medical applications (such as the prevention of post-surgical adhesions and scars, wound coverings, artificial pericardia, tissue reconstruction and programmed drug delivery) (Guda et al. 2000). Subsequently, expression of the human somatotropin via the tobacco chloroplast genome (Staub et al. 2000) to high levels (7% of total soluble protein) was observed. The following investigations that are in progress illustrate the power of this technology to express small peptides, entire operons, vaccines that require oligomeric proteins with stable disulfide bridges and monoclonals that require assembly of heavy/light chains via chaperonins. It is essential to develop a selection system free of antibiotic resistant genes for the edible insulin approach to be successful. One such marker free chloroplast transformation system has been accomplished (Daniell et al. 2000). Experiments are in progress to develop chloroplast transformation of edible leaves (alfalfa and lettuce) for the practical applications of this approach.

Please amend the first full paragraph of page 11 as follows:

(60/185,987) GVGVP (SEQ ID NO:20) is a PBP made from synthetic genes. At lower temperatures the polymers exist as more extended molecules which, on raising the temperature above the transition range, hydrophobically fold into dynamic structures called β-spirals that further aggregate by hydrophobic association to form twisted filaments (Urry, 1991; Urry, et al., 1994). Inverse temperature transition offers several advantages. Expense associated with chromatographic resins and equipment are

eliminated. It also facilitates scale up of purification from grams to kilograms. Milder purification conditions use only a modest change in temperature and ionic strength. This also facilitates higher recovery, faster purification and high volume processing. Protein purification is generally the slow step (bottleneck) in pharmaceutical product development. Through exploitation of this reversible inverse temperature transition property, simple and inexpensive extraction and purification is performed. The temperature at which the aggregation takes place can be manipulated by engineering biopolymers containing varying numbers of repeats and changing salt concentration in solution (McPherson et al., 1996). Chloroplast mediated expression of insulin-polymer fusion protein eliminates the need for the expensive fermentation process as well as reagents needed for recombinant protein purification and downstream processing.

Please amend paragraph 2 on page 12 as follows:

(60/185,987) In accordance with one advantageous feature of this invention, we use poly(GVGVP) (SEQ ID NO:1) as a fusion protein to enable hyper-expression of insulin and accomplish rapid one step purification of fusion peptides utilizing the inverse temperature transition properties of this polymer. In another advantageous feature of this invention, we develop insulin-CTB fusion protein for oral delivery in nicotine free edible tobacco (LAMD 605). Both features are accomplished as follows:

Please amend paragraph 3 on page 12 as follows:

a) Develop recombinant DNA vectors for enhanced expression of Proinsulin as fusion proteins with GVGVP (SEQ ID NO:20) or CTB via chloroplast genomes of tobacco,

Please amend paragraph 2 on page 14 as follows:

(60/185,987) Protein Based Polymers (PBP): The synthetic gene that codes for a bioelastic PBP was designed after repeated amino acid sequences GVGVP (SEQ ID NO:20), observed in all sequenced mammalian elastin proteins (Yeh et al. 1987). Elastin

is one of the strongest known natural fibers and is present in skin, ligaments, and arterial walls. Bioelastic PBPs containing multiple repeats of this pentamer have remarkable elastic properties, enabling several medical and non-medical applications (Urry et al. 1993, Urry 1995, Daniell 1995). GVGVP (SEQ ID NO:20) polymers prevent adhesions following surgery, aid in reconstructing tissues and delivering drugs to the body over an extended period of time. North American Science Associates, Inc. reported that GVGVP (SEQ ID NO:20) polymer is non-toxic in mice, non-sensitizing and non-antigenic in guinea pigs, and non-pyrogenic in rabbits (Urry et al. 1993). Researchers have also observed that inserting sheets of GVGVP (SEQ ID NO:20) at the sites of contaminated wounds in rats reduces the number of adhesions that form as the wounds heal (Urry et al. 1993). In a similar manner, using the GVGVP (SEQ ID NO:20) to encase muscles that are cut during eye surgery in rabbits prevents scarring following the operation (Urry et al. 1993, Urry 1995). Other medical applications of bioelastic PBPs include tissue reconstruction (synthetic ligaments and arteries, bones), wound coverings, artificial pericardia, catheters and programmed drug delivery (Urry, 1995; Urry et al., 1993, 1996).

Please amend paragraph 3 on page 14 as follows:

(60/185,987) We have expressed the elastic PBP $(GVGVP)_{121}$ (SEQ ID NO:21) in *E. coli* (Gutda et al. 1995, Brixey et al. 1997), in the fungus *Aspergillus nidulans* (Herzog et al. 1997), in cultured tobacco cells (Zhang et al. 1995), and in transgenic tobacco plants (Zhang et al. 1996). In particular, $(GVGVP)_{121}$ (SEQ ID NO:21) has been expressed to such high levels in *E. coli* that polymer inclusion bodies occupied up to about 90% of the cell volume. Also, inclusion bodies have been observed in chloroplasts of transgenic tobacco plants (see attached article, Daniell and Gudya, 1997). Recently, we reported stable transformation of the tobacco chloroplasts by integration and expression of the biopolymer gene (EG121), into the Large Single Copy region (5,000 copies per cell) or the Inverted Repeat region (10,000 copies per cell) of the chloroplast genome (Guda et al., 1999).

Please amend paragraph 2 on page 15 as follows:

(60/185,987) Accordingly, one achievement according to this invention is to use poly(GVGVP) (SEQ ID NO:1) as a fusion protein to enable hyper-expression of insulin and accomplish rapid one step purification of the fusion peptide. At lower temperatures the polymers exist as more extended molecules which, on raising the temperature above the transition range, hydrophobically fold into dynamic structures called β-spirals that further aggregate by hydrophobic association to form twisted filaments (Urry, 1991). Through exploitation of this reversible property, simple and inexpensive extraction and purification is performed. The temperature at which aggregation takes place (T_1) is manipulated by engineering biopolymers containing varying numbers of repeats or changing salt concentration (McPherson et al., 1996). Another group has recently demonstrated purification of recombinant proteins by fusion with thermally responsive polypeptides (Meyer and Chilkoti, 1999). Polymers of different sizes have been synthesized and expressed in *E. coli.* This approach also eliminates the need for expensive reagents, equipment and time required for purification.

Please amend paragraph 2 on page 16 as follows:

(60/185,987) The [[E]]expression and assembly of CTB in transgenic potato tubers has been reported (Arakawa et al. 1997). The CTB gene including the leader peptide was fused to an endoplasmic reticulum retention signal (SEKDEL) (SEQ ID NO:2) at the 3' end to sequester the CTB protein within the lumen of the ER. The DNA fragment encoding the 21-amino acid leader peptide of the CTB protein was retained to direct the newly synthesized CTB protein into the lumen of the ER. Immunoblot analysis indicated that the plant derived CTB protein was antigenically indistinguishable from the bacterial CTB protein and that oligomeric CTB molecules (Mr ~ 50 kDa) were the dominant molecular species isolated from transgenic potato leaf and tuber tissues. Similar to bacterial CTB, plant derived CTB dissociated into monomers (Mr ~ 15 kDa) during heat acid treatment.

Please amend the second partial paragraph on page 20 as follows:

(60/185,987) Recently, the human pre-proinsulin gene was obtained from Genentech, Inc. First, the pre-proinsulin was sub-cloned into pUC19 to facilitate further manipulations. The next step was to design primers to make chloroplast expression vectors. Since we are interested in proinsulin expression, the 5' primer was designed to land on the proinsulin sequence. This FW primer eluded the 69 bases or 23 coded amino acids of the leader or pre-sequence of preproinsulin. Also, the forward primer included the enzymatic cleavage site for the protease factor Xa to avoid the use of cyanogen bromide. Beside the Xa-factor, a SmaI site was introduced to facilitate subsequent subcloning. The order of the FW primer sequence is SmaI – Xa-factor – Proinsulin gene. The reverse primer includes BamHI and XbaI sites, plus a short sequence with homology with the pUC19 sequence following the proinsulin gene. The 297bp PCR product (Xa Pris) includes three restriction sites, which are the SmaI site at the 5'-end and XbaI/BamHI sites at the 3' end of the proinsulin gene. The Xa-Pris was cloned into pCR2.1 resulting in pCR2.1 – Xa- Pris (4.2kb). Insertion of Xa-Pris into the multiple cloning site of pCR2.1[[,]] resulted in additional flanking restriction enzyme sites that will be used in subsequent sub-cloning steps. A GVGVP 50-mer (SEQ ID NO:22) was generated as described previously (Daniell et al. 1997). The ribosome binding sequence was introduced by digesting pUCs-10, which contains the RBS sequence GAAGGAG (SEQ ID NO:23), with NooI and Hind III flanking sites. The plasmid pUC19-50 was also digested with the same enzymes. The 50mer gene was eluted from the gel and ligated to pUCs-10 to produce pUCs-10-50mer. The ligation step inserted into the 50mer gene a RBS sequence and a SmaI site outside the gene to facilitate subsequent fusion to proinsulin.

Please amend paragraph 3 on page 23 as follows:

(60/185,987) It is evident that the insulin-polymer fusion proteins are stable in *E. coli.* Confirming this observation, recently another lab has shown that the PBP polymer protein conjugates (with thioredoxin and tendamistat) undergo thermally reversible phase

transition, retaining the transition behavior of the free polymer (Meyer and Chikoti, 1999). These results clearly demonstrate that insulin fusion has not affected the inverse temperature transition property of the polymer. One of the concerns is the stability of insulin at temperatures used for thermally reversible purification. Temperature induced production of human insulin has been in commercial use (Schmidt et al. 1999). Also, the temperature transition can be lowered by increasing the ionic strength of the solution during purification of this PSP (McPherson et al, 1996). Thus, GVGVP-fusion (SEQ ID NO:20) could be used to purify a multitude of economically important proteins in a single inexpensive step.

Please amend the first full paragraph on page 24 as follows:

(60/263,668) The gel was first stained with 0.3M $CuCl_2$ and then the same gel was stained with Commassie R-250 Staining Solution for an hour and then destained for 15 min first, and then overnight. $CuCl_2$ creates a negative stain (Lee et al. 1987). Polymer proteins (without fusion) appear as clear bands against a blue background in color or dark against a light semiopaque background as shown in Fig. 12A. This stain was used because other protein stains such as Coomassie Blue R250 does not stain the polymer protein due to the lack of aromatic side chains (McPherson et al., 1992). Therefore, the observation of the 24 kDa protein in R250 stained gel as shown in Fig. 12B is due to the insulin fusion with the polymer. This observation was further confirmed by probing these blots with the anti-human proinsulin antibody. As anticipated, the polymer insulin fusion protein was observed in western blots as shown in Figs. 13A and B. Larger proteins observed in Figs. 13A – C are tetramer and hexamer complexes of proinsulin. It is evident that the insulin-polymer fusion proteins are stable in *E. coli.* Confirming this observation, recently others have shown that the PBP polymer protein conjugates (with thioredoxin and tendamistat) undergo thermally reversible phase transition, retaining the transition behavior of the free polymer (Meyer and Chilkoti, 1999). These results clearly demonstrate that insulin fusion has not affected the inverse temperature transition property of the polymer. One of the concerns is the stability of

insulin at temperatures used for thermally reversible purification. Temperature induced production of human insulin has been in commercial use (Schmidt et al. 1999). Also, the temperature transition can be lowered by increasing the ionic strength of the solution during purification of this PBP (McPherson et al, 1996). Thus, GVGVP-fusion (SEQ ID NO:20) could be used to purify a multitude of economically important proteins in a simple inexpensive step.

Please amend the second full paragraph on page 25 as follows:

(60/185,987) Protease Xa Digestion of the Biopolymer-proinsulin fusion protein and Purification of Proinsulin: Factor Xa was purchased from New England Biolabs at a concentration of 1.0 mg/ml. The Factor Xa is supplied in 20mM HEPES, 500 mM, NaCl, 2mM $CaCl_2$, 50% glycerol[[,]] (pH 8.0). The reaction was carried out in a 1:1 ratio of fusion protein to reaction buffer. The reaction buffer was made with 20mM Tris-HCl, 100 mM NaCl, 2mM $CaCl_2$[[,]] (pH 8.0). The enzymatic cleavage of the fusion protein to release the proinsulin protein from the $(GVGVP)_{40}$ (SEQ ID NO:24) was initiated by adding the protease to the purified fusion protein at a ratio (ww) of approximately 1,500. This digestion was continued for 5 days with mild stirring at 4°C. Cleavage of the fusion protein was monitored by SDS-PAGE analysis. After the cleavage, the same conditions are used for purification of the proinsulin protein. The purification steps are the same as for the purification of the fusion protein, except that instead of recovering the pellet, the supernatant is saved. We detected cleaved proinsulin in the extracts isolated in 6M guanidine hydrochloride buffer as shown in Fig. 1C 11. Conditions can be estimized for complete cleavage. The Xa protease has been successfully used to cleave $(GVGVP)_{20}$-GST fusion (SEQ ID NO:25) (McPherson et al. 1992). Therefore, cleavage of proinsulin from GVGVP (SEQ ID NO:20) using the Xa protease does not pose problems.

Please amend paragraph 3 on page 25 as follows:

(60/263,668) The enzymatic cleavage of the fusion protein to release the proinsulin protein from the $(GVGVP)_{40}$ (SEQ ID NO:24) was initiated by adding the

factor 10A protease to the purified fusion protein at a ratio (w/w) of approximately 1:500. Cleavage of the fusion protein was monitored by SDS-PAGE analysis. We detected cleaved proinsulin in the extracts isolated in 6M guanidine hydrochloride buffer as shown in Figs. 13A and B. Conditions are noweing optimized for complete cleavage. The Xa protease has been successfully used previously to cleave $(GVGVP)_{20}$-GST fusion (SEQ ID NO:25) (McPherson et al. 1992).

Please amend paragraph 3 on page 26 as follows:

(60/185,987) Vector for CTB expression in chloroplasts: The leader sequence (63 bp) of the native CTB gene (372 bp) was deleted and a start codon (ATG) introduced at the 5' end of the remaining CTB gene (309 bp). Primers were designed to introduce a rbs site 5 bases upstream of the start codon. The 5' primer (38mer) was designed to land on the start codon and the 5'-end of the CTB gene. This primer had an Xbal site at the 5'-end, the rbs site [GGAGG] (SEQ ID NO:26), a 5 bp breathing space followed by the first 20 bp of the CTB gene. The 3' primer (32mer) was designed to land on the 3' end of the CTB gene and it introduced restriction sites at the 3' end to facilitate subcloning. The 347 bp rCTB PCR product was subcloned into pCR2.1 resulting in pcCR2.1-rCTB. The final step was insertion of rCTB into the Xbal site of the universal or tobacco vector (pLB-CtV2) that allows the expression of the construct in *E. coli* and chloroplasts. Restriction enzyme digestion of the pLD-LH-rCTB vector with BamH1 was performed to confirm the correct orientation of the inserted fragment in the vector.

Please amend the first full paragraph on page 29 as follows:

(60/185,987) CTB-Proinsulin Vector Construction: The chloroplast expression vector pLD-CTB-Proins was constructed as follows. First, both proinsulin and cholera toxin B-subunit genes were amplified from suitable DNA using primer sequences. Primer 1 contains the GGAGG (SEQ ID NO:26) chloroplast preferred ribosome binding site five nucleotides upstream of the start codon (ATG) for the CTB gene and a suitable restriction enzyme site (Spel) for insertion into the chloroplast vector. Primer 2 eliminates the stop

codon and adds the first two amino acids of a flexible hinge tetrapeptide GPGP (SEQ ID NO:3) as reported by Bergerot et al. (1997), in order to facilitate folding of the CTB-proinsulin fusion protein. Primer 3 adds the remaining two amino acids for the hinge tetrapeptide and eliminates the pre-sequence of the pre-proinsulin. Primer 4 adds a suitable restriction site (Spel) for subcloning into the chloroplast vector. Amplified PCR products were inserted into the TA cloning vector. Both the CTB and proinsulin PCR fragments were excised at the Smal and Xbal restriction sites. Eluted fragments were ligated into the TA cloning vector. Interestingly, all white colonies showed the wrong orientation for CTB insert while three of the five blue colonies examined showed the right orientation of the CTB insert. The CTB-proinsulin fragment was excised at the EcoRl sites and inserted into EcoRl digested dephosphorolated pLD vector. Resultant onicroplast integration expression vector, pLD-CTB-Proins will be tested for expression in *E. coli* by western blots. After confirmation of expression of CTB-proinsulin fusion in *E. coli*, pLD-CTB-Proins will be bombarded into tobacco cells as described below.

Please amend paragraph 2 on page 30 as follows:

(Update "Human Insulin") Another parameter of foreign protein production to be investigated is post-translational. The DNA for the putative chaperonin in the *Bacillus thuringiensis* Cry 2A2 operon encodes a protein that could potentially fold and crystallize CTB-Proinsulin, which would allow it to accumulate in large quantities protected from chloroplast proteases and facilitate in subsequent purification. Standard molecular biology techniques were used to insert this DNA fragment immediately upstream of the 5'UTR of the construct containing the chloroplast optimized proinsulin. Additionally, another vector was constructed to contain only Shine-Dalgarno sequence (GGAGG) (SEQ ID NO:26) followed by the sequence encoding for the Cholera toxin B subunit and synthetic chloroplast optimized proinsulin fusion (CTB-PTpris). This construct will allow us to determine the value of the proinsulin sequence modification both with and without the 5'UTR.

Please amend the first full paragraph on page 34 as follows:

(60/185,987) Optimization of fusion gene expression: It has been reported that foreign genes are expressed between 5% (cry1AC, cry11A) and 30% (uldA) in transgenic chloroplasts (Daniell, 1999). If the expression levels of the CTB-Proinsulin or polymer-proinsulin fusion proteins are low, several approaches will be used to enhance translation of these proteins. In chloroplast, transcriptional regulation of gene expression is less important, although some modulations by light and developmental conditions are observed (Cohen and Mayfield, 1997). RNA and protein stability appear to be less important because of observation of large accumulation of foreign proteins (e.g. GUS up to 30% of total protein) and tps1 transcripts 16,966-fold higher than the highly expressing nuclear transgenic plants. Chloroplast gene expression is regulated to a large extent at the post-transcriptional level. For example, 5' UTRs are used for optional translation of chloroplast mRNAs. Shine-Delgarno (GGAGG) (SEQ ID NO:26) sequences as well as a stem-loop structure located 5' adjacent to the SD sequence are used for efficient translation. A recent study has shown that insertion of the psbA 5' UTR downstream of the 16S rRNA promoter enhanced translation of a foreign gene (GUS) hundred-fold (Eibl et al. 1999). Therefore, the 85-bp tobacco chloroplast DNA fragment (1595 - 1680) containing 5' psbA UTR will be amplified using the following primers (SEQ ID NOs:6 and 7, respectively): cctttaaaaagccttccattttctattt, gccatggtaaaatcttggtttatta. This PCR product will be inserted downstream of the 16S rRNA promoter to enhance translation of the proinsulin fusion proteins.

Please amend paragraph 2 on page 34 as follows:

(60/185,987) Yet another approach for enhancement of translation is to optimize codon compositions of these fusion protein. Since both fusion proteins are expressed well in *E. coli*, we expected efficient expression in chloroplasts. However, optimizing codon compositions of proinsulin and CTB genes to march the psbA gene could further enhance the level of translation. Although rbcL (RuBisCO) is the most abundant protein on earth, it is not translated as frequently as the psbA gene due to the extremely high

turnover of the psbA gene product. The psbA gene is under stronger selection for increased translation efficiency and is the most abundant thylakoid protein. In addition, codon usage in higher plant chloroplasts is biased towards the NNC codon of 2-fold degenerate groups (i.e. TTC over TTT, GAC over GAT, CAC over CAT, AAC over AAT, ATC over ATT, ATA etc.). This is in addition to a strong bias towards T at third position of 4-fold degenerate groups. There is also a context effect that should be taken into consideration while modifying specific codons. The 2-fold degenerate sites immediately upstream from a GNN codon do not show this bias towards NNC, (TTT GGA is preferred to TTC GGA while TTC CGT is preferred to TTT CGT, TTC AGT to TTT AGT and TTC TCT to TTT TCT) ~~(SEQ ID NO:8)~~. In addition, highly expressed chloroplast genes use GNN more frequently than other genes. The web site _____ may be used to optimize codon composition by comparing different species. Abundance of amino acids in chloroplasts can be taken into consideration (pathways compartmentalized in plastids as opposed to those that are imported into plastids).

Please amend paragraph 2 on page 35 as follows:

(60/263,668) We have reported that foreign genes are expressed between 3% (*cry2Aa2*) and 46% (*cry2Aa2* operon) in transgenic chloroplasts (Kota et al. 1999; De Cosa et al. 2001). Several approaches may be used to enhance translation of the recombinant proteins. In chloroplasts, transcriptional regulation as a bottle-neck in gene expression has been overcome by utilizing the strong constituitive promoter of the 16s rRNA (Prrn). One advantage of Prrn is that it is recognized by both the chloroplast encoded RNA polymerase and the nuclear encoded chloroplast RNA polymerase in tobacco (Allison et al. 1996). Several investigators have utilized Prrn in their studies to overcome the initial hurdle of gene expression, transcription (De Cosa et al. 2001, Eibl et al. 1999, Staub et al. 2000). RNA stability appears to be one among the least problems because of observation of excessive accumulation of foreign transcripts, at times 16,966-fold higher than the highly expressing nuclear transgenic plants (Lee et al. 2000). Also,

other investigations regarding RNA stability in chloroplasts suggest that efforts for optimizing gene expression need to be addressed at the post-transcriptional level (Higgs et al. 1999, Eibl et al. 1999). Our work focuses on addressing protein expression post-transcriptionally. For example, 5' and 3' UTRs are needed for optimal translation and mRNA stab[[l]]ility of chloroplast mRNAs (Zerges 2000). Optimal ribosomal binding sites (RBS's) as well as a stem-loop structure located 5= adjacent to the RBS are needed for efficient translation. A recent study has shown that replacement of the Shine-Delgarno (GGAGG) (SEQ ID NO:26) with the psbA 5' UTR downstream of the 16S rRNA promoter enhanced translation of a foreign gene (GUS) hundred-fold (Eibl et al. 1999). Therefore, the 200-bp tobacco chloroplast DNA fragment (1680-1480) containing 5' psbA UTR may be used. This PCR product is inserted downstream of the 16S rRNA promoter to enhance translation of the recombinant proteins.

Please amend the first full paragraph on page 52 as follows:

A remarkable feature of chloroplast genetic engineering is the observation of exceptionally large accumulation of foreign proteins in transgenic plants, as much as 46% of CRY protein in total soluble protein, even in bleached old leaves (3). Stable expression of a pharmaceutical protein in chloroplasts was first reported for GVGVP (SEQ ID NO:20), a protein based polymer with varied medical applications (such as the prevention of post-surgical adhesions and scars, wound coverings, artificial pericardia, tissue reconstruction and programmed drug delivery (88)). Subsequently, expression of the human somatotropin via the tobacco chloroplast genome (9) to high levels (7% of total soluble protein) was observed. The following investigations that are in progress in the Daniell laboratory illustrate the power of this technology to express small peptides, entire operons, vaccines that require oligomeric proteins with stable disulfide bridges and monoclonals that require assembly of heavy/light chains via chaperonins.

Please amend paragraph 3 on page 56 as follows:

The first vector included the gene that codes for the mature HSA plus an additional ATG as a translation initiation codon. We included the ATG in one of the primers of the PCR, 5 nucleotides downstream of the chloroplast preferred RBS sequence GGAGG (SEQ ID NO:26). The cDNA sequence of the mature HSA (cloned in Dr. Mingo-Castel's laboratory) was used as a template. The PCR product was cloned into PCR 2.1 vector, excised as an EcoRI-NotI fragment and introduced into the pLD vector. (Update "Human Therapeutic Proteins") The vector includes the chloroplast preferred Ribosome Binding Site (RBS) sequence GGAGG (SEQ ID NO:26).

Please amend paragraph 3 on page 63 as follows:

Foreign genes are expressed between 3% (*cry2Aa2*) and 47% (*cry2Aa2* operon) in transgenic chloroplasts (3,85). Based on the outcome of the evaluation of HSA chloroplast transgenic plants, several approaches can be used to enhance translation of the recombinant proteins. In chloroplasts, transcriptional regulation of gene expression is less important, although some modulations by light and developmental conditions are observed (123). RNA stability appears to be one among the least problems because of observation of excessive accumulation of foreign transcripts, at times 16,966-fold higher than the highly expressing nuclear transgenic plants (124). Chloroplast gene expression is regulated to a large extent at the post-transcriptional level. For example, 5' UTRs are necessary for optimal translation of chloroplast mRNAs. Shine-Dalgarno (GGAGG) (SEQ ID NO:26) sequences, as well as a stem-loop structure located 5' adjacent to the SD sequence, are required for efficient translation. A recent study has shown that insertion of the psbA 5' UTR downstream of the 16S rRNA promoter enhanced translation of a foreign gene (GUS) hundred-fold (125a). Therefore, the 200-bp tobacco chloroplast DNA fragment (1680-1480) containing 5' psbA UTR should be used. This PCR product is inserted downstream of the 16S rRNA promoter to enhance translation of the recombinant proteins.